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Research Article

Isolation, identification and evaluation of potential biocontrol agents against major cankers in apple

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ABSTRACT: Twenty three fungi of 15 genera, 2 bacteria and one actinomycete were isolated from pink (*Corticium salmonicolor*), smoky blight (*Sphaeropsis malorum*) and stem brown (*Dothiorella mali*) canker of apple stems and twigs. Among these, *Trichoderma viride, Trichothecium roseum, Alternaria* spp., *Aspergillus* sp., *Penicillium* sp., *Fusarium sp., Diplodia* sp. and actinomycete, were frequently encountered. *In vitro* evaluation of various microorganisms for their antagonistic activity revealed *Trichoderma viride*-2 was most effective in inhibiting the growth of pink (87.6%), smoky blight (88.4%) and stem brown (84.0%) canker followed by *T. longibrachiatum*-1, *T. harzianum*-1 and *T. hamatum* respectively. Amongst non-*Trichoderma* species *Fusarium lateritium* was the most effective. *Bacillus subtilis* provided maximum growth inhibition of the three cankers *in vitro*. *T. viride* isolate-2 was effective in preventing the formation of new canker lesions under laboratory conditions. Similar results in healing canker lesions were also obtained under field conditions by applying the antagonists as spore suspension as well as slurry preparations.

KEY WORDS: Antagonists, Apple, bacteria, biocontrol agents, canker, microflora, *Trichoderma*

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INTRODUCTION

In apple, pink (*Corticium salmonicolor* Berk and Br.), smoky blight (*Sphaeropsis malorum* Berk.), and stem brown (*Dothiorella mali* H. Jacks.) cankers are the major bottlenecks in successful cultivation and occurrence of these cankers is responsible for early decline of established orchards (Sharma and Bhardwaj, 1999). Spray of fungicides (carbendazim, captan or copper oxychloride) at late dormancy and post harvest stage along with local applications of Chaubatia or Bordeaux paint have been reported effective against these major cankers (Anon., 2006), but they are cost prohibitive and pollutive besides resulting in residual toxicity in plants and development of resistance in pathogens (Sharma and Bhardwaj, 2002). Though a little information is available only from abroad on biological control of apple canker caused by *Nectria galligena,* and *Chondrostereum purpreum* (Groscloude *et al*., 1974; Swinburne, 1978), these cankers are not at all serious in apple in India. The present studies were therefore undertaken to isolate various microflora associated with canker lesions in endemic areas and evaluate them under laboratory and field conditions to select the potential ones for their further use.

MATERIALS AND METHODS

Isolation and identification of canker pathogens

Target pathogens of pink, smoky blight and stem brown cankers were isolated by taking the infected bits from canker lesions. These were surface sterilized in rectified alcohol for one minute, then placed on potato-dextrose agar medium and incubated at 25±1°C. The respective cultures of these pathogens were purified by hyphal tip as well as single spore method and were stored for further use. The pathogenicity of these fungal pathogens was proved by twig inoculation method (Borecki and Milliikan, 1969). The target pathogens were identified on the basis of morphology and spore characteristics (Barnett, 1962; Tims, 1963; Gupta and Agarwala, 1973).

Isolation and identification of microflora associated with canker lesions

The canker affected branches/ twigs of cultivar "Royal Delicious" were collected from different Apple Research Orchard of Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni – Solan; Pajhota and Churvadhar

(Rajgarh) in Sirmour district; Seobagh, Raison and Banjar in Kullu district and Kotkhai in Shimla district of Himachal Pradesh. These samples were collected during post rainy season during September–October.

The microorganisms from in and around apple cankers viz., *C. salmonicolor* (pink canker), *S. malorum* (smoky blight canker) and *D. mali* (stem brown) affected portion were isolated by host tissue segment (5 No. for each canker) and serial dilution methods and their axenic cultures were maintained (Dhingra and Sinclair, 1985). The isolated microorganisms were identified on the basis of cultural and morphological characters as described in different manual and monographs (Rifai, 1969; Booth, 1971; Samuels, 1996).

In vitro *evaluation of microorganisms against canker pathogens*

Dual culture technique

Isolated nineteen fungal microorganisms viz., three isolates [Tv1 (Nauni), Tv2 (Sirmour), Tv3 (Kullu)] of *Trichoderma viride,* two each of *T. longibrachiatum* [Tl1 (Nauni), Tl2 (Kullu)] and *T. harzianum* [Th1 (Nauni), Th2 (Sirmour)], *T. hamatum, T. polysporum* [Tp1 (Kullu), Tp2 (Kotkhai)], *Fusarium lateritium, Fusarium sp., Trichothecium roseum, Cladosporium sp., Chaetomium sp., Aspergillus sp., Aureobasidium sp* were separately evaluated *in vitro* against three major pathogens viz., *C. salmonicolor*, *S. malorum* and *D. mali* by dual culture technique (Dennis and Webster, 1971). Antagonistic activity of bacteria (*Bacillus subtilis*: procured from Department of Mycology & Plant Pathology, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan, *Bacillus sp., Pseudomonas sp.*) was studied by streak plate method (Utkhede and Rahe, 1983). Per cent growth inhibition for each target fungal pathogen was calculated by following the method of Vincent (1947). I=C-T/C x 100, where I is Inhibition of fungal growth, C is fungal growth in control and T is fungal growth in the treatment.

In vitro *excised twig method*

Healthy apple twigs (6-8 x 1-1.5 cm) were cut and individually inoculated by following the twig inoculation method developed by Borecki and Millikan (1969) and incubated at 24±1°C. In pre-inoculation, the antagonist's bits five mm were put three days before the pathogen inoculation, whereas in post–inoculation, antagonists were applied after three days of target pathogen inoculation. In control each pathogen was separately inoculated. The lesion size developed for both pre and post inoculation experiments was measured after 21st day of inoculation when excised twig were fully covered with growth of pathogen and turned brown to slightly blackish.

Field evaluation of potential biocontrol agents against target cankers

The efficacy of six BCAs was further ascertained under field conditions by adopting following two methods.

Spore suspension method

Surface growth of individual BCA on potato dextrose agar medium (5 Petri plates) was scrapped after seven days of incubation at 25±1°C and was suspended in 500 ml sterilized distilled water supplemented with two per cent jaggery. The final concentration of each BCA was adjusted to 3 x 10^6 cfu ml^{-1}. It was filtered through double layered sterile muslin cloth.

Slurry method

Six biocontrol agents were separately cultured on potato dextrose broth by inoculating aseptically 500ml broth with four bits of 5mm size of each antagonist and were incubated at 25±1°C for 14 days. The broth culture was homogenised in a blender for 2-3 minutes and was mixed in malt extract in 1: 2 ratio (v/w) finally to make slurry.

Evaluation

Field trials on evaluation of BCAs against pink canker was laid out in cv. Royal Delicious growing in the apple orchard of Department of Fruit Breeding and Genetic Resources, Dr. Y. S. Parmer University of Horticulture and Forestry, Nauni, Solan for two consecutive years 2007-08. The canker lesions were carefully scrapped off with sharp edged knife in the month of January (27-29) and spore suspension (3 x 10^6 cfu ml^{-1}.) of each BCA was applied separately with automiser, whereas slurry was applied with spatula. The treated lesions were immediately covered with moist cotton and thereafter also adequate moist conditions were maintained for 7 days by using sterile water. In control treatment BCAs were not applied. Similar trials against smoky blight and stem brown canker were also laid at Rajgarh and Seobagh, respectively. For individual canker, each treatment was replicated four times. Data on lesion size were recorded before application of BCAs in the month of Jan. and 10 months later in Nov. Per cent healing in lesion size for each BCA was calculated by adopting the formula (LSBA-LSAA)÷LSBA×100 where LSBA = lesion size before application of BCA, LSAA = lesion size 10 months after BCAs applications. The extent of callusing around the canker lesion was measured and designated as 0 mm (–) = No callusing, + = 0.1-5.00 mm callusing, ++ = 5.01-10 mm callusing, +++ = > 10 mm callusing.

Experimental design and statistical analysis

The experiments were laid in randomized block design and each treatment was replicated four times. Data recorded for different years were pooled for each target canker and were subjected to statistical analysis by following the method of variance described by Gomez and Gomez (1984) to find out the least significant difference (LSD) amongst the treatments at 5 per cent level.

RESULTS AND DISCUSSION

Twenty three different fungi namely; *T. viride*, *T. longibrachiatum, T. harzianum*, *T. hamatum, T. polysporum, Aureobasidium* sp., *Alternaria alternata, Alternaria* sp., *Trichothecium roseum, Rhizopus* sp., *Aspergillus niger, Aspergillus* sp., *Penicillium glaucum, Penicillium* sp., *Coniothyrium fuckelii, Phoma glomerata, Monilinia* sp., *Fusarium lateritium, Fusarium* sp., *Cladosporium sp., Coryneum* sp., *Diplodia* sp., *Chaetomium* sp., two bacteria viz., *Pseudomonas and Bacillus* sp. and one actinomycete were isolated from canker affected portion of the apple branches and twigs. Of these *T. viride, T. roseum, A. alternata, Alternaria* sp., *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Diplodia* sp. and actinomycete were present at three locations (Nauni, Rajgarh, Kullu), whereas others were obtained from either one or two locations. Swinburne (1973) and Razdan and Putto (2002) obtained similar microflora from aerial plant surfaces of apple, stone and nut fruits. Swinburne (1973) also reported that *Trichoderma* species occurred more frequently than others during repeated isolations. Though *Trichoderma* species occur abundantly in the apple orchards as reported above, they fail to establish and provide protection against canker due to 7-8 sprays of fungicides during the crop season to control various fungal diseases. These repeated sprays either kill or reduce the population of *Trichoderma* species and hence may not provide the expected control. All the micro-organisms isolated from infected lesions inhibited the mycelial growth of target pathogens (Table 1). *T. viride*-2 exhibited maximum inhibition of mycelial growth of *C. salmonicolor*, *S. malorum* and *D. mali* followed by *T. longibrachiatum*-1 and *T. harzianum*-1 in that order. Other isolates/ species of *Trichoderma* as well as *F. lateritium* inhibited the growth of these pathogens to a greater extent in comparison to other fungal flora while minimum inhibition was recorded with *Chaetomium* sp. (30.1%).

Pre-inoculation application of *T. viride*-2 was individually most effective against all the three cankers caused by *C. salmonicolor, S. malorum* and

Table 1. *In vitro* evaluation of different microorganisms against major apple canker pathogens

Microorganism	Mean radial growth inhibition (%)*		
	CS	SM	DM
Trichoderma viride–1	84.9 (67.22)[$]	82.8 (65.55)	72.9 (58.64)
Trichoderma viride –2	87.6 (69.43)	88.4 (70.16)	84.0 (66.45)
Trichoderma viride –3	79.1 (62.84)	76.9 (61.31)	76.2 (60.84)
Trichoderma longibrachiatum–1	87.2 (69.06)	84.4 (66.78)	80.4 (63.74)
Trichoderma longibrachiatum –2	67.5 (55.25)	65.1 (53.82)	75.8 (60.56)
Trichoderma harzianum–1	86.3 (68.37)	84.0 (66.45)	78.7 (62.55)
Trichoderma harzianum –2	73.6 (59.09)	72.9 (58.64)	69.9 (56.18)
Trichoderma hamatum	83.3 (65.92)	81.5 (64.55)	73.7 (59.18)
Trichoderma polysporum–1	76.9 (61.31)	80.2 (63.64)	76.8 (61.22)
Trichoderma polysporum–2	76.8 (61.21)	82.8 (65.50)	77.2 (61.48)
Fusarium lateritium	67.7 (55.41)	71.5 (57.75)	63.6 (52.90)
Bacillus subtilis	73.0 (58.73)	78.0 (62.07)	78.1 (62.11)
Pseudomonas spp.	57.9 (49.56)	58.6 (49.96)	63.1 (52.69)
Control	90.0**	90.0*	90.0*
LSD at 5 per cent level	0.91	0.87	1.16

[$] = Figures in parentheses are arc sine transformed values; * = mean of 4 replications; ** = radial growth of fungus in control; CS = *Corticium salmonicolor*, SM = *Sphaeropsis malorum*, DM = *Dothiorella mali*

Table 2. Evaluation of antagonists against major apple cankers by excised twig method

Antagonists	Pre–inoculation Av. lesion size (cm)			Post–inoculation Av. lesion size (cm)		
	CS	SM	DM	CS	SM	DM
T. viride-2	0.59	0.54	0.51	1.09	1.04	1.10
T. longibrachiatum-1	0.60	0.58	0.55	1.13	1.07	1.13
T. harzianum -1	0.64	0.62	0.59	1.15	1.07	1.16
T. hamatum	0.65	0.65	0.62	1.21	1.16	1.25
T. polysporum-2	0.71	0.72	0.62	1.25	1.53	1.28
F. lateritium	0.75	0.81	0.71	1.29	1.19	1.27
Control	3.10	3.00	2.87	3.11	2.69	3.26

LSD at 5 per cent level: Pre-inoculation, post-inoculation due to antagonists = 0.11, 0.16, canker pathogens = 0.08, 0.11; Antagonists' × Canker pathogens = 0.24, 0.31

Table 3. Field evaluation of antagonists against major apple cankers applied as spore suspension

Antagonists	Recovery (%) of canker lesions			Callus formation		
	CS	SM	DM	CS	SM	DM
T. viride-2	49.2 (44.53)*	43.2 (41.09)	39.6 (39.02)	+++	+++	+++
T. longibrachiatum-1	45.6 (42.46)	35.6 (36.63)	33.1 (35.12)	+++	+++	++
T. harzianum -1	44.1 (44.62)	34.2 (35.79)	30.6 (33.58)	++	+++	++
T. hamatum	41.1 (39.87)	28.9 (32.51)	24.4 (29.60)	++	++	+
T. polysporum-2	42.5 (40.66)	31.9 (34.34)	35.9 (36.81)	++	++	++
F. lateritium	31.1 (33.88)	26.5 (30.94)	24.3 (29.53)	++	++	+
CD	1.32	2.12	1.92			

CD at 5 per cent level: due to antagonists = 2.11; canker pathogens = 1.13; antagonists' × canker pathogens = 3.24; CS = *Corticium salmonicolor*, SM = *Sphaeropsis malorum*, DM = *Dothiorella mali*; *figures in parentheses are arc sine transformed values

Table 4. Field evaluation of antagonists against major apple canker applied as slurry method

Antagonists	Recovery (%) of canker lesions			Callus formation		
	CS	SM	DM	CS	SM	DM
T. viride-2	51.4 (45.80) *	45.3 (42.30)	40.8 (39.70)	+++	+++	+++
T. longibrachiatum-1	50.2 (45.11)	41.4 (40.05)	39.2 (38.76)	+++	+++	+++
T. harzianum -1	49.9 (44.94)	39.8 (39.11)	34.1 (35.73)	+++	+++	+++
T. hamatum	43.5 (41.27)	32.6 (34.82)	22.1 (28.04)	++	+++	+
T. polysporum-2	44.1 (41.61)	35.2 (36.39)	36.4 (37.11)	+++	+++	++
F. lateritium	35.3 (36.45)	26.9 (31.24)	20.8 (27.13)	++	++	+

CD at 5 per cent level: due to antagonists = 2.45; canker pathogens = 1.38; antagonists × canker pathogens = 3.52; CS = *Corticium salmonicolor*, SM = *Sphaeropsis malorum*, DM = *Dothiorella mali*; *figures in parentheses are arc sine transformed values

D. mali in inhibiting the lesion size (Table 2). It restricted their lesion size to 0.59, 0.54 and 0.51 cm in comparison to 3.1, 3.0 and 2.87 cm formed in untreated control. *T. longibrachiatum*-1, *T. harzianum*-1 and *T. hamatum* were ont par with *T. viride-2*. *T. polysporum* -2 and *F. lateritium* comparatively were less effective but also provided significantly higher inhibition of canker lesions. Similar results in limiting the lesion size caused by target pathogens by respective antagonist were also observed in post-inoculation excised twig method but the pre-inoculation treatment was superior in restricting the lesion size due to their preventive action.

Application of spore suspension (3 x 10^6 cfu ml^{-1}) of *T. viride*-2 exhibited maximum recovery of lesions pink, smoky blight and stem brown cankers to an extent of 49.2, 43.2 and 39.6 per cent, respectively and was closely followed by *T. longibrachiatum*-1 and *T. harzianum*-1 in that order (Table 3). Next best results were obtained with *T. polysporum*-2, followed by *T. hematum* whereas *F. lateritium* was least effective. *T. viride -2, T. longibrachiatum*-1 and *T. harzianum*-1 were superior in formation the callus around the canker lesions. Although similar results (Table 4) were also obtained in slurry application of antagonists but it provided slightly higher recovery (~3-7%) of lesions because slurry provided better support to antagonists for an early establishment. Application of biocontrol agents in the present study also provided higher recovery as compared to carbendazim paint, which exhibited 25.7 and 32.6 per cent healing of pink and smoky blight cankers, respectively (Sharma and Ram, 2010).

In vitro and field evaluation of biocontrol agents against pink, smoky blight and stem brown cankers in apple revealed that *T. viride*-2 was the most effective and was closely followed by *T. longibrachiatum*-1 and *T. harzianum*-1 in order. *T. viride* has also been found effective in controlling *Valsa* (*V. ceratosperma*) canker (Sawamura *et al*., 1990). Stefanova *et al.* (1999) reported that *T. viride* and *T. longibrachiatum* produced significant inhibition zone against *Phytophthora nicotianae and Rhizoctonia solani* under laboratory conditions through production of non-volatile antifungal metabolites and lytic enzymes. Molecules produced by *Trichoderma* species have potential for promoting plant growth, induction of systemic resistance and exhibiting antagonism against various pathogens and nematodes (Yedidia *et al*., 1999; Sharon *et al*., 2001). Enhanced recovery of canker lesion obtained may be due to the multifaceted activity of *Trichoderma* species in comparison to systemic fungicidal paint presently in practice. The above mentioned research reports support the present findings and therefore the native isolates of *Trichoderma* species found effective against the three major cankers of apple in the present study can be further used as an input/ component in integrated disease management strategy.

REFRENCES

Anonymous, 2006. *Package of Practices*. Directorate of Extension Education, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India, 54 pp.

Barnett, H. L. 1962. *Illustrated genera of imperfect fungi*. 2nd Ed. Burgess Publishing Company, Minneapolis 15, Minnesota. USA, 225 pp.

Booth, C. 1971. The genus *Fusarium.* Mc Graw and Hill Company. London, UK, 273 p.

Borecki, Z. and Millikan, D. F. 1969. A rapid method for determining pathogenicity of *Phytophthora cactorum*. *Phytopathology,* **59**: 247–248.

Dennis, C. and Webster, J. 1971. Antagonism properties of species groups of *Trichoderma*. III. Hyphal interactions. *Transactions of the British Mycological Society*, **57**: 363–369.

Dhingra, O. D. and Sinclair, J. B. 1985. *Basic Plant Pathology Methods*. CRC Press, Boca Raton, Florida, USA, 355 pp.

Gomez, K. A. and Gomez, A. A. 1984. *Statistical Procedures for Agricultural Research*, 2nd Ed. John Wiley and Sons, New York, USA, 680 pp.

Grosclaude, C., Dubos, V. and Ricard, J. L. 1974. Antagonism between ungerminated spores of *Trichoderma viride* and *Stereum purpureum*. *Plant Disease Reporter*, **58**: 71–74.

Gupta, G. K. and Agarwala, R. K. 1973. Canker diseases of apple in Himachal Pradesh. *Indian Journal of Mycology and Plant Pathology,* **3**: 189–192.

Razdan, V. K. and Putto, B. L. 2002. Antagonism of *Wilsonomyces carpophilus* causing shot hole of stone and nut fruits by phylloplane microflora. *Plant Disease Research,* **17**: 256–260.

Rifai, M. A. 1969. A revision of genus *Trichoderma*. *Mycology Paper,* **116**: 29pp.

Samuels, G. J. 1996. *Trichoderma;* a review of biology and systematics of the genus. *Mycological Research*, **100**: 923–935.

Sawamura, K., Harda, Y and Yukika, K. 1990. Apple valsa canker caused by *Valsa ceratosperma*. Tode: Fr. Maire. *Bulletin of the faculty of Agriculture, Hirosaki University,* **53**: 10–32.

Sharma, I. M. and Bhardwaj, S. S. 1999. Canker and foliar diseases of apple, pp 15-53. In: Verma, L. R. and Sharma, R. C. (Eds.). *Diseases of Horticultural Crops: Fruits*. Indus Publishing Co. New Delhi, India, 724 pp.

Sharma, I. M. and Bhardwaj, S. S. 2002. Canker disease in apple: Present status and future strategies, pp 323-329. In: Singh, J. M., Sharma, R. P. and Sharma, V. K. (Eds.). *Horti-Vision 2020*. State Department of Horticulture, Shimla, Himachal Pradesh, India.

Sharma, I. M. and Ram, V. 2010. Occurrence and management of important cankers in apple. *Journal of Mycology and Plant Pathology*, **40**: 213–218.

Sharon, E., Bar-E-Yal, M., Chet, I., Herrerastrellat, A., Kleifeld, O. and Spiegel, Y. 2001. Biocontrol of root knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*. *Phytopathology,* **91**: 687– 693.

Stefanova, M. A., Leiva, L., Larrinaga, M. and Coronado, F. 1999. Metabolic activity of *Trichoderma* spp. isolates for a control of soilborne phytopathogenic fungi. *Review Faculty of Agronomy* (LUZ), **16**: 509–516.

Swinburne, T. R. 1973. Microflora of apple leaf scars in relation to infection by *Nectria galligena. Transactions of the British Mycological Society,* **60**: 389–403.

Swinburne, T. R. 1978.The potential value of bacterial antagonists for the control of apple cankers. *Annals of Applied Biology,* **77**: 94–96.

Tims, E. C. 1963. *Corticium salmonicolor* in the United State. *Plant Disease Reporter,* **47**: 1055–1059

Utkhede, R. S. and Rahe, J. E. 1983. Interactions of antagonists and pathogens in biological control of onion white rot. *Phytopathology,* **73**: 890.

Yedidia, I., Benhamou, N. and Chet, I. 1999. Induction of defense response in cucumber plants (*Cucumis sativus* L.) by biocontrol agent *Trichoderma harzianum*. *Applied and Environmental Microbiology*, **65**: 1061–1070.

Vincent, J. H. 1947. Distortion of fungal hyphae in the presence of certain inhibitor. *Nature*, **15**: 580.